12

PATENT COOPERATION TREATY

REC'D 04 JAN 2002

WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GWS/21524	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB00/03519	International filing date *(day/month/year)* 13/09/2000	Priority date *(day/month/year)* 13/09/1999
International Patent Classification (IPC) or national classification and IPC C07K14/33		
Applicant MICROBIOLOGICAL RESEARCH AUTHORITY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand 07/03/2001	Date of completion of this report 28.12.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Strobel, A Telephone No. +49 89 2399 7362

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

   Description, pages:

   1-19 as originally filed

   Claims, No.:

   1-21 as received on 07/12/2001 with letter of 06/12/2001

   Drawings, sheets:

   1/5-5/5 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-21
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-21
Industrial applicability (IA)	Yes:	Claims	1-21
	No:	Claims	

2. Citations and explanations
   see separate sheet

Re Item I

Basis of the opinion

The claimed priority has not been checked since the International Search Report does not cite documents published after the priority date.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: US-A-5 601 826

D2: GIMENEZ ET AL.: JOURNAL OF PROTEIN CHEMISTRY, vol. 12, no. 3, 1993, pages 351-363, XP000986491

1. **Amendments**

The amended set of claims fulfils the requirements of Article 34(2)(b) PCT.

2. **Inventive step**

D2 describes mixtures of proteolytic fragments of C.botulinum Neurotoxin (NT) A (these fragments are toxin derivatives). The 97 and 132 kDa fragments as well as the mixture of the 132 and 18 kDa fragments show very low toxicity (mouse lethality) relative to the NT since they lack the receptor binding moiety of integral NT. D2 evokes the possibility that said toxicity is either due to the presence of trace amounts of NT contamination in the sample or that these fragments possess a low intrinsic toxicity, without giving preference to any of these mechanisms. The effect of the subject-matter of claim 1 relative to D2 is that said residual toxicity of a toxin derivative preparation is reduced. The underlying technical problem of claim 1 is thus the provision of a method for reducing the toxicity of a toxin preparation, the solution being the incubation of the derivative preparation with a ligand which selectively binds to the toxin but not to the toxin derivative.

D1 describes antibodies directed against linear epitopes of the C-terminal half of the Heavy Chain of C.tetani NT. It is known in the prior art that this C-terminal half contains the receptor binding activity of clostridial NT which is responsible of the binding of the NT to their target cells.

The man skilled in the art confronted with the problem of reducing toxicity of a

toxin derivative preparation would readily combine the teaching of D2 with that of D1. Both documents belong to the same technical field, namely clostridial NTs. Even if D2 presents two possible explanations for the toxicity associated with preparations of clostridial NT fragments, the man skilled in the art would inevitably incubate the fragment preparation of D2 with the antibody of D1.
Thus, claims 1-21 are obvious (Non-fulfilment of Article 33(3) PCT).

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 14 May 2001 (14.05.01)	
International application No. PCT/GB00/03519	**Applicant's or agent's file reference** GWS/21524
International filing date (day/month/year) 13 September 2000 (13.09.00)	**Priority date** (day/month/year) 13 September 1999 (13.09.99)
Applicant CHADDOCK, John, Andrew et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   07 March 2001 (07.03.01)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Pascal Piriou Telephone No.: (41-22) 338.83.38

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/19863 A1

(51) International Patent Classification[7]: C07K 14/33, A61K 39/08

(21) International Application Number: PCT/GB00/03519

(22) International Filing Date: 13 September 2000 (13.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9921592.3 13 September 1999 (13.09.1999) GB

(71) Applicant (*for all designated States except US*): MICROBIOLOGICAL RESEARCH AUTHORITY [GB/GB]; CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): CHADDOCK, John, Andrew [GB/GB]; Microbiological Research Authority CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). ALEXANDER, Frances, Celine, Gail [GB/GB]; Microbiological Research Authority CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). FOSTER, Keith, Alan [GB/GB]; Microbiological Research Authority CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(74) Agents: SCHLICH, George, William et al.; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

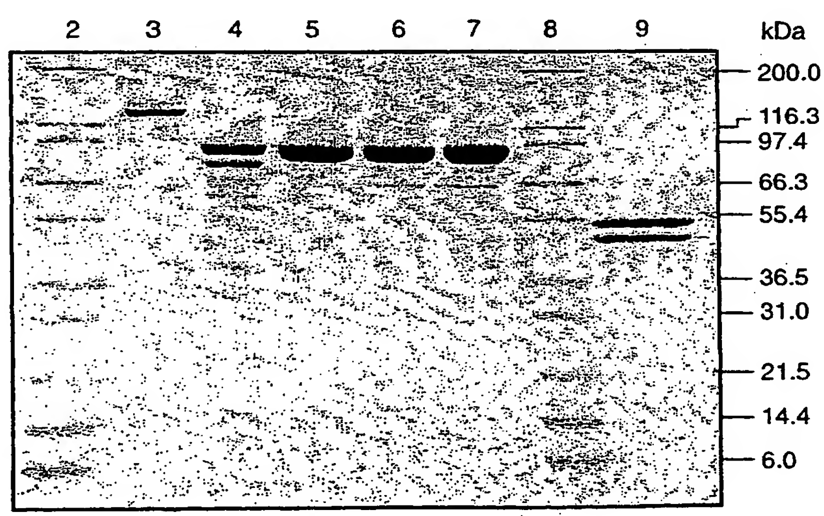
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: PREPARATION OF HIGHLY PURE TOXIN FRAGMENTS

SDS-PAGE analysis of LH_N/A purified by immunoaffinity approach

A



(57) Abstract: Toxin derivatives are made by proteolytic treatment of holotoxin, and the toxicity of a toxin derivative preparation is reduced by contacting the preparation with a ligand, which can be a metal or an antibody or another ligand. This ligand selectively binds to the toxin but not to the toxin derivative. Removing the ligand and toxin bound to the ligand further reduces toxicity. Compositions containing the purified derivative, optionally plus the toxin and the ligand are described.

WO 01/19863 A1

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PREPARATION OF HIGHLY PURE TOXIN FRAGMENTS

This invention relates to derivatives, such as fragments, of toxins, particularly clostridial neurotoxins. It also relates to preparations containing those derivatives and to methods of obtaining the derivatives and the preparations.

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of bacterium of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There at present eight neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C_1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesised by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa.

The clostridial neurotoxins bind to an acceptor site (Black, J. D. & Dolly, J. O., Neuroscience, **23**, 767-779, 1987 and Dolly *et al.* in Cellular and Molecular Basis of Cholinergic Function, ed. Dowdall, M. J. & Hawthorne, J. N., Chapter 60, 1987) on the cell membrane of the motoneurone at the neuromuscular junction and are internalised by an endocytotic mechanism (Montecucco *et al.*, Trends Biochem. Sci., **11**, 314, 1986). It is believed that the clostridial neurotoxins are highly selective for motoneurons due to the specific nature of the acceptor site on those neurones. The binding activity of clostridial neurotoxins is known to reside in a carboxy-terminal region of the heavy chain component of the dichain neurotoxin molecule, a region known as H_C. The N-terminal region of the H-chain (H_N domain) is thought to be of central importance in the translocation of the L-chain

into the cytosol and has been demonstrated to from channels in lipid vesicles (Shone *et al*, Eur. J. Biochem. **167**, 175-180).

Clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity that is known to reside in the L-chain. Each toxin serotype hydrolyses a specific peptide bond within one of three proteins of the SNARE complex; VAMP (synaptobrevin), syntaxin or SNAP-25. Proteolytic cleavage of one of these proteins leads to instability of the SNARE complex and consequent prevention of vesicular release. The enzymatic activity of the light chain of the neurotoxin leads to inhibition of neurotransmitter release, which results in a prolonged muscular paralysis.

The central role of the SNARE proteins in regulated secretion has been convincingly established (e.g. Niemann *et al.*, (1994) *Trends Cell Biol.*, **4**, 179-185). However, the correlation of SNARE protein involvement with the release of specific hormones, peptides, transmitters and other signalling molecules remains to be established in the majority of cases. The range of highly specific endopeptidase activities of clostridial neurotoxin serotypes provides a unique approach to the understanding of SNARE-mediated events. Unfortunately, the use of native clostridial toxins for the study of such events is limited by at least two important aspects. Firstly, the expression of the requisite toxin receptor is restricted to a limited population of cells, thereby limiting the range of cell types in which SNARE-mediated events can be studied without cellular disruption. Secondly, the significant hazards associated with working with potent neurotoxins lead to restrictions on the range of applications and experimental design. Clostridial neurotoxins are the most potent neuroparalytic toxins known and must be manipulated in specialised laboratory conditions by specially trained and, preferably, vaccinated staff. The ability to produce highly purified non-toxic fragments of clostridial neurotoxins possessing the enzymatic activity of the clostridial neurotoxins and capable of delivery to the cytosol of selected cells would therefore provide a valuable tool for

studying secretory mechanisms.

The ability of the clostridial neurotoxins' enzymatic activity to destabilise SNARE complex formation and thereby inhibit vesicle fusion at the plasma membrane also has therapeutic potential. A number of therapeutic applications have been proposed (e.g. WO 96/33273 & WO 94/21300) that are dependent on the successful retargeting of clostridial neurotoxin fragments. These approaches require a source of non-hazardous neurotoxin fragment that is suitable for the synthesis of non-toxic conjugates, since the side effect profile of a therapeutic contaminated with neurotoxin would be unacceptably high. In addition to retargeting of clostridial toxin fragments, there are further applications for non-toxic clostridial derivatives. For example, as an immunogen for vaccine preparation, as a source of material from which highly purified neurotoxin-related fragments can be prepared, and as a non-toxic endopeptidase standard in diagnostic kits (e.g. WO 95/33850).

Since the cell binding function of clostridial neurotoxins resides in the H_C domain of the heavy chain, generation of a fragment in which the binding capability of the H_C has been deleted but the properties of the H_N domain are retained (LH_N) is potentially a suitable method for the production of a non-toxic derivative.

It is known to prepare these fragments by proteolytic treatment of toxin and then separation of toxin from fragments by anion exchange chromatography, and such methods successfully yield fragments that are 99.99% pure.

A central or recurrent problem associated with obtaining or using products from toxins made by these methods is the risk of residual toxicity in those products. It would hence be desirable to provide a method of removing toxin from such products. However, existing protocols have reached the limits of their abilities in this respect.

For example, it has been observed that the known fragments often exhibit a high inhibition of neurotransmitter release by neuronal cells *in vitro*. This has hampered investigation into the properties of conjugates in which a toxin fragment is combined with a ligand providing a specific targeting function, because of difficulty in providing controls against which to judge the conjugate activity.

It is an object of the present invention to provide a method of preparing a toxin derivative preparation. A further object of the invention is to provide a method of removing toxin from a toxin derivative preparation.

It has been discovered in accordance with the present invention that existing toxin derivative preparations, though considered to be pure, and though containing toxin at an extremely low level, nevertheless contain sufficient residual toxin to interfere with the applications of the fragment, conjugate or other toxin derivative.

Thus, in a first aspect of the invention, there is provided a method of reducing toxicity of a toxin derivative preparation, comprising contacting said preparation with a ligand which selectively binds to the toxin but not to the toxin derivative.

In a use of the invention, the ligand binds to and effectively neutralises residual toxin which is contaminating the toxin derivative preparation. The ligand preferentially binds to the toxin compared with its binding to the

derivative. Thus in use there may be some small loss of derivative at the same time as toxin binds to the ligand. It has been found that prior art preparations of toxin derivatives may contain toxin at levels of one toxin molecule per 10,000 toxin fragments, and even at this low level the toxin adversely effects the uses of the fragment. According to the invention, an antibody that preferentially binds to the toxin, but not to the derivative, can be used to reduce or remove toxicity associated with the toxin, thereby enabling the effects and applications of the derivative to be examined and used without any masking effect of the residual toxin.

In an embodiment of the invention, described in more detail below, a toxin fragment prepared according to prior art methods significantly inhibited substance P release from dorsal root ganglia. This inhibition was reduced almost entirely by the combination of the fragment with an antibody that specifically bound to toxin.

Antibodies for use in the present invention can be prepared using polyclonal or monoclonal techniques. Suitable methodology is found for example in "Antibodies: A laboratory manual, by Ed Harlow and David Lane, 1988". Monoclonal antibodies can be prepared by immunising mice against toxin, harvesting lymphocyte cells from the spleens of immunised mice and fusing these with myeloma cells. Antibodies secreted by the resulting hybridomas are screened for binding to toxin and positive clones selected and propagated. Monoclonal antibodies are harvested from cultured hybridomas and purified using chromatographic methods - see for example Pharmacia handbook on "Monoclonal antibody purification". As an alternative to immunising mice with the toxin itself, the mice can be immunised with a different source of a H_c domain, whether obtained from native material or expressed in an alternative, non clostridial, host. Alternatively, mice can be immunised with toxoided preparations of intact neurotoxins and the anti Hc antibodies selected. This may be achieved in at least 2 ways for example: specific Hc antisera may be bound to

immobilised Hc and subsequently eluted for use. Conversely, specific Hc antisera may be obtained by adsorbing non-Hc antisera onto LH_N.

This invention thus provides, in specific examples, preparation of specific, defined fragments from native clostridial neurotoxins. Purified clostridial neurotoxin obtained from *Clostridia sp.* using previously reported techniques (for example, Shone, C. C. and Tranter, H. S. (1995) *in* "Clostridial Neurotoxins - The molecular pathogenesis of tetanus and botulism", (Montecucco, C., Ed.), pp. 152-160, Springer) can be fragmented by proteolytic or chemical cleavage to yield a crude mixture of derivatives that possess elements of the light chain, heavy chain or both (Gimenez, J. A. & DasGupta, B. R. (1993) J. Protein Chem., **12**, 351-363; Shone, C. C., Hambleton, P. and Melling, J. (1987) *Eur. J. Biochem.* **167**, 175-180). Classical chromatographic techniques are used to separate the crude mixture into partially purified fragments, the residual toxicity of which would make them unsuitable for many applications, and the fragments are then combined with a neutralizing ligand.

During testing by the inventors of a conjugate or a toxin fragment with a targeting ligand, it was not possible *in vitro* to determine the selectivity of an LH_N fragment, though the reasons for this were not known to the inventors at the time. According to the invention, it has surprisingly been found that very low levels of toxins present were masking the LH_N fragment activity, and now advantageously it is possible further to reduce this toxin content so that pure LH_N activity can be measured and assessed.

In the particular case of *in vivo* uses of fragments and conjugates and other derivatives, a specific embodiment of the invention, described in more detail below, determined that fragments according to the present invention exhibited a level of toxicity that was more than 10 times lower than that of the prior art fragments, which prior art fragments were hitherto considered as being pure and toxin-free.

It is a preferred additional step in the method of the invention to separate the ligand from the toxin derivative preparation.

In one embodiment of the invention, a specific binding event between an immobilised matrix and a domain present on the neurotoxin, but absent from the fragment, is used to separate neurotoxin from the fragment. Such a method is used specifically to bind neurotoxin and other fragments possessing the requisite binding domain from a crude neurotoxin fragment mixture. Non-binding fragments that are free of neurotoxin are simply isolated from the column flow through. Binding fragments including neurotoxin can be isolated by altering the conditions of binding, for instance by altering the chemical environment (e.g. pH, ionic strength) or incorporation of a substance that competes for the binding site (e.g. peptide, sugar moiety). One suitable example is a column containing the natural receptor, or a version of the receptor, for which the neurotoxin has an affinity. This receptor may be purified and immobilised to a matrix for use in a column or free in solution, or present in a preparation of cells or cell membrane, and is of use for purification of LH_N. Typically, a crude preparation of clostridial neurotoxin fragments is applied to the receptor preparation to bind neurotoxin and other fragments that possess receptor binding properties. Non-binding fragments, which include LH_N, are recovered from the receptor preparation by simple elution. Binding fragments and neurotoxin are released and harvested as described above.

Purification of clostridial endopeptidases is also suitably achieved according to the invention using metal ion chromatography. Clostridial endopeptidases are characterised as metalloendopeptidases due to the co-ordination of a metal ion at the active site of the enzyme. Given this specific metal ion binding capacity of clostridial neurotoxins, it might be predicted that both neurotoxin and endopeptidase fragments bind to immobilised metal resin via this catalytic site interaction. However, it is found that the LH_N does not bind to the chelating column whereas the

neurotoxin does. In an example, set out in more detail below, a method for the purification of LH_N/A utilises immobilised zinc ions to bind BoNT/A and purify LH_N/A. The low toxicity of LH_N/A purified by this method is also confirmed below.

Another embodiment of the invention comprises immobilising specific antibodies to a column resin. The antibodies are selected on the basis of their specificity for epitopes present on the neurotoxin but absent on the desired fragment, exemplified by LH_N below. In the presence of partially purified LH_N, antibodies with specificity for the H_C domain will only bind to the neurotoxin. Contaminating neurotoxin is removed from the LH_N preparation by entrapment on the immobilised-antibody matrix, whereas LH_N, which is not recognised by the antibodies, does not interact with the column. This method is surprisingly efficient at removing residual toxicity from the LH_N preparation and affords an effective purification technique. Entrapment of the neurotoxin contaminant by antibody binding, rather than specifically binding the LH_N, enables the elution conditions to be maintained at the optimum for LH_N stability. The use of harsh elution conditions e.g. low pH, high salt, chaotropic ions, which may have detrimental effects on LH_N polypeptide folding and enzymatic activity, are therefore avoided. Neurotoxin and other binding fragments may be eluted from the antibody column to release clostridial neurotoxin derivatives that are purified from derivatives deficient in the H_C domain epitopes.

An additional advantage of the methods of the invention is thus that the desired component of the mixture that is being purified, that is to say the fragment or the conjugate or the other derivative, is that portion which is eluted from the column whereas the undesired portion, the toxin, remains bound to the column. This has the benefit that the desired material is less affected by the column and that no additional step, for example to elute bound, desired material from the column, is required as part of the method.

Further embodiments of the invention use two different affinity techniques in combination. In a preferred embodiment of the invention exemplified below, combinations of antibodies with different epitope recognition properties are used. By utilising monoclonal antibodies with different recognition epitopes, small conformational changes in neurotoxin can be accommodated. In this way, a greater proportion of neurotoxin is targeted for removal from the crude starting mixture.

It is further preferred that the method of the invention comprises an additional step, after separating the ligand from the toxin derivative preparation, which ligand we will refer to in these present paragraphs as the first ligand, of contacting the toxin derivative preparation with a second ligand, which selectively binds to the first ligand but not to the toxin derivative. It is occasionally the case that ligand attached, for example, to a chromatography column, detaches and thus the toxin derivative elutes from the column in combination with complexes of ligand and toxin. This opens the possibility of separation of the complex at a future time, releasing the toxin. It is an advantage of the preferred method of the invention that these ligand-toxin complexes, if present in the toxin derivative preparation are substantially removed by use of the further ligand.

The further, or second, ligand can suitably be an antibody that binds to the antibody used as the first ligand. A specific embodiment of the invention thus comprises:-

> preparing a toxin derivative preparation which comprises toxin derivative which is contaminated by low levels of toxin;

> contacting the preparation with a ligand which selectively binds to the toxin but not to the toxin derivative;

separating the ligand from the toxin derivative preparation, thereby separating toxin from the toxin derivative preparation;

contacting the preparation with a further ligand that binds selectively to the first ligand, or binds selectively to a complex of the first ligand with toxin; and

separating the second ligand from the toxin derivative preparation.

The first ligand can be an antibody which binds to an H_c portion of the toxin, and the second ligand can be a further antibody or immunoglobulin binding domain which binds to the first antibody or to a complex of the first antibody with toxin, and in a specific example the second ligand can be protein G. In one particular example of the preferred embodiment of the invention in use, injection of 20 micrograms LH_N purified according to the invention using the first ligand resulted in 0 out of 4 mice surviving, whereas use of the second ligand to remove antibody - toxin complexes resulted in 4 out of 4 survivors. This very surprising result exhibits the improved purity of the toxin derivative following application of the second purification step.

Specific antibodies or antibody fragments are optionally mixed with partially purified clostridial neurotoxin fragments in solution to form bound antibody-neurotoxin complexes. The antibody-neurotoxin complexes are then isolated from the mixture (e.g. by Protein G chromatography) and removed to yield purified agents in solution.

Immobilised monoclonal antibodies may be used specifically to bind contaminating neurotoxin and intact H_C or other contaminants. Two BoNT/A H_C specific monoclonal antibodies are thus utilised in a method that is surprisingly efficient at binding contaminating neurotoxin to prepare LH_N/A of high purity and low toxicity.

The present invention is of application to any toxins which can be converted into useful fragments, conjugates and other derivatives, for example by proteolytic action, and is of particular application to clostridial neurotoxins, especially botulinum and tetanus toxins of all sub-groups.

By toxin derivative, it is intended to encompass all derivatives of a toxin that are prepared directly or indirectly from native toxin, for example by proteolytic action on the toxin, which methods can result in a preparation having residual, low levels of toxin present. Thus, a toxin derivative preparation according to the definition does not include recombinant toxin derivatives which are guaranteed to be free of toxin. The meaning of derivative thus encompasses toxin fragments and conjugates of toxin fragments with other molecules as well as variants of toxins and toxin fragments and conjugates.

In a further aspect, the invention provides an affinity chromatography column, for removal of toxin from a toxin derivative preparation, wherein the column comprises a ligand that selectively binds to toxin but not to the toxin derivative. This column is of use in separating toxin from toxin derivative, and the ligand employed is suitably an antibody or a metal ion.

A still further aspect of the invention lies in a toxin derivative preparation comprising 1-100 ppm toxin per toxin derivative, preferably 10 - 100ppm. This preparation is derived from native toxin, and has a greatly reduced residual level of toxin.

A yet further aspect of the invention provides a composition comprising a derivative of a toxin and a pharmaceutically acceptable carrier, and further comprising a ligand that binds selectively to the toxin. The composition may for example comprise a conjugate of a toxin with a ligand that binds selectively to the toxin, wherein the toxin is bound non-covalently to the ligand. The toxin is thus neutralized by the ligand.

The invention is now described in specific embodiments, accompanied by drawings in which:-

Fig. 1 shows SDS-PAGE analysis of LH_N/A purified by the immunoaffinity approach;

Fig. 2 shows retention of functional activity of LH_N/A prepared by the invention

Fig. 3 shows inhibition of neurotransmitter release from eSCN by LH_N/A and BoNT/A; and

Fig. 4 shows mouse toxicity data for purified LH_N/A.

Example 1

<u>Inhibition of Glycine Release</u>

Embryonic spinal cord cells were treated for one day in the presence of 30 micrograms per ml of LH_N/A (prior art preparation). This treatment resulted in an inhibition of glycine release of 64%. A parallel treatment was carried out in the presence of antibody 5BA 9.3, which binds specifically to botulinum neurotoxin A. This resulted in an inhibition of glycine release which was reduced to 44%.

Example 2

<u>Inhibition of Substance P Release</u>

Embryonic Dorsal route ganglia were treated for 3 days with 20 micrograms per ml LH_N/A, LH_N/A+ antibody 5BA 9.3 and also with a conjugate of LH_N/A with a targeting ligand in the presence of antibody 5BA 9.3.

The inhibition of substance P release was significant when the 20μg/ml

LH_N/A fragment was used alone, and was at a level of about 26%. This level was reduced to about 4% in the presence of the specific antibody, and the level rose to about 21% when the conjugate (also 20μg/ml) was used in the presence of the specific antibody.

Example 3

Production of LH_N/A from BoNT/A by antibody-affinity chromatography

BoNT/A was prepared according to a previous method (Shone, C. C. and Tranter, H. S. (1995) *in* "Clostridial Neurotoxins - The molecular pathogenesis of tetanus and botulism", (Montecucco, C., Ed.), pp. 152-160, Springer). FPLC® chromatography media and columns were obtained from Amersham Pharmacia Biotech, UK. Affi-gel Hz™ matrix and materials were from BioRad, UK.

Preparation of an anti-BoNT/A antibody-affinity column

An antibody-affinity column was prepared with specific monoclonal antibodies essentially as suggested by the manufacturers' protocol. Briefly, monoclonal antibodies 5BA2.3 & 5BA9.3 which have different epitope recognition in the H_C domain (Hallis, B., Fooks, S., Shone, C. and Hambleton, P. (1993) *in* "Botulinum and Tetanus Neurotoxins", (DasGupta, B. R., Ed.), pp. 433-436, Plenum Press, New York) were purified from mouse hybridoma tissue culture supernatant by Protein G (Amersham Pharmacia Biotech) chromatography. These antibodies represent a source of BoNT/A H_C-specific binding molecules and can be immobilised to a matrix or used free in solution to bind BoNT/A. In the presence of partially purified LH_N/A (which has no H_C domain) these antibodies will only bind to BoNT/A. The antibodies 5BA2.3 & 5BA9.3 were pooled in a 3:1 ratio and two mg of the pooled antibody was oxidised by the addition of sodium periodate (final concentration of 0.2%) prior coupling to 1ml Affi-Gel Hz™ gel (16 hours at room temperature). Coupling efficiencies were routinely

greater than 65%. The matrix was stored at 4°C in the presence of 0.02% sodium azide.

Purification strategy for the preparation of pure LH_N/A

BoNT/A was treated with 17μg trypsin per mg BoNT/A for a period of 72-120 hours. After this time no material of 150kDa was observed by SDS-PAGE and Coomassie blue staining. The trypsin digested sample was chromatographed (FPLC® system, Amersham Pharmacia Biotech) on a Mono Q® column (HR5/5) to remove trypsin and separate the majority of BoNT/A from LH_N/A. The crude sample was loaded onto the column at pH 7 in 20mM HEPES, 50mM NaCl and 2ml LH_N/A fractions eluted in a NaCl gradient from 50mM to 150mM. The slightly greater pI of BoNT/A (6.3) relative to LH_N/A (5.2) encouraged any BoNT/A remaining after trypsinisation to elute from the anion exchange column at a lower salt concentration than LH_N/A. LH_N/A containing fractions (as identified by SDS-PAGE) were pooled for application to the antibody column.

The semi-purified LH_N/A mixture was applied and reapplied at least 3 times to a 1-2ml immobilised monoclonal antibody matrix at 20°C. After a total of 3 hours in contact with the immobilised antibodies, the LH_N/A-enriched supernatant was removed. Entrapment of the BoNT/A contaminant, rather than specifically binding the LH_N/A, enables the elution conditions to be maintained at the optimum for LH_N stability. The use of harsh elution conditions e.g. low pH, high salt, chaotropic ions, which may have detrimental effects on LH_N polypeptide folding and enzymatic activity, are therefore avoided. Treatment of the immobilised antibody column with 0.2M glycine/HCl pH2.5 resulted in regeneration of the column and elution of BoNT/A-reactive proteins of 150kDa.

The LH_N/A enriched sample was then applied 2 times to a 1ml HiTrap® Protein G column (Amersham Pharmacia Biotech) at 20°C. Protein G was

selected since it has a high affinity for mouse monoclonal antibodies. This step was included to remove BoNT/A-antibody complexes that may leach from the immunocolumn. Antibody species bind to the Protein G matrix allowing purified LH_N/A to elute.

The profile of the purification procedure is illustrated by the SDS-PAGE analysis in Figure 1. Trypsin digested BoNT/A (lane 4) was applied to a Mono Q® anion exchange column and fractions harvested (lane 5). Material pre- and post-Protein G is indicated in lanes 6 and 7 respectively. Lane 7 represents the final purified LH_N/A preparation. Samples were analysed by SDS-PAGE on 4-20% polyacrylamide and stained with Coomassie blue (Panel A), or Western blotted and probed with anti-BoNT/A (Panel B). Molecular weight markers are indicated on the Figure with reference to the standards in lanes 2 & 8. Molecular weight markers in lane 1 are compatible with enhanced chemiluminescence and are for visualisation purposes only.

In vitro SNAP-25 peptide cleavage

The *in vitro* cleavage of SNAP-25 by LH_N/A and other endopeptidase samples were assessed essentially as described previously (Hallis, B., James, B. A. F. and Shone, C. C. (1996) *J. Clin. Microbiol.* **34**, 1934-1938). Figure 2 clearly demonstrates the similarity in catalytic activity between purified LH_N/A and reduced BoNT/A. A series of LH_N/A (●) and BoNT/A (□) dilutions were incubated for 1 hour prior to assessment of SNAP-25 cleavage. The data is representative of at least 7 experiments which have suggested EC_{50} for LH_N/A and BoNT/A to be 3.8 ± 0.7pM and 3.6 ± 0.6pM respectively.

SDS-PAGE and Western Blotting

SDS-PAGE and Western Blotting were performed using standard protocols.

Proteins were resolved on a 4-20% Tris/glycine polyacrylamide gel (Novex) and either stained by the addition of Coomassie blue or transferred to nitrocellulose. Positive binding of antibodies was detected by enhanced chemiluminescence.

Example 4

Production of LH_N/A from BoNT/A by
<u>immobilised metal affinity chromatography</u>

BoNT/A was prepared according to a previous method (Shone, C. C. and Tranter, H. S. (1995) *in* "Clostridial Neurotoxins - The molecular pathogenesis of tetanus and botulism", (Montecucco, C., Ed.), pp. 152-160, Springer). FPLC® chromatography media and columns were obtained from Amersham Pharmacia Biotech, UK. Affi-gel Hz™ matrix and materials were from BioRad, UK.

Preparation of cationic metal affinity column

An affinity column was prepared essentially as suggested by the manufacturers' protocol. Briefly, chelating Sepharose® (Amersham Pharmacia Biotech) was washed with 50mM EDTA + 1M NaCl (10 column volumes), washed with 10 column volumes ultra-high purity water, primed with 5mg/ml $ZnCl_2$ (10 column volumes –neutral pH, filtered), and finally washed with purified water (10x column volumes).

Purification strategy for the preparation of pure LH_N/A

BoNT/A was treated with 17μg trypsin per mg BoNT/A for a period of 72-120 hours. After this time no material of 150kDa was observed by SDS-PAGE and Coomassie blue staining. The trypsin digested sample was chromatographed (FPLC® system, Amersham Pharmacia Biotech) on a Mono Q® column (HR5/5) to remove trypsin and separate the majority of BoNT/A

from LH_N/A. The crude sample was loaded onto the column at pH 7 in 20mM HEPES, 50mM NaCl and 2ml LH_N/A fractions eluted in a NaCl gradient from 50mM to 150mM. The slightly greater pI of BoNT/A (6.3) relative to LH_N/A (5.2) encouraged any BoNT/A remaining after trypsinisation to elute from the anion exchange column at a lower salt concentration than LH_N/A. LH_N/A containing fractions (as identified by SDS-PAGE) were pooled for application to the metal affinity column.

For the purification of 1mg LH_N/A a 1 ml column of Zn^{2+} primed chelating Sepharose®, prepared as described above was equilibrated to the appropriate pH (7.5-8.5) using 50mM HEPES + 1M NaCl ('equilibration buffer'). 1 mg of LH_N (post Mono Q® fractionation) was applied to the column after dialysis against the appropriate equilibration buffer. Material that did not bind was recovered and the column washed with excess equilibration buffer. Bound material was eluted by the application of equilibration buffer supplemented with 10mM EDTA.

Another suitable method is described by Rossetto et al, Biochem J., (1992), vol. 285, pp 9-12.

Example 5

Evaluation of BoNT/A contamination levels in LH_N/A prepared by <u>immunocolumn chromatography in primary cultures of spinal cord neurons</u>

Inhibition of [^{3}H]-glycine release from eSCN

Spinal cord neurons are exquisitely sensitive to neurotoxin, with an IC_{50} of inhibition of glycine release of 0.027 ± 0.0006pM after 3 days exposure to BoNT/A (unpublished observations). Therefore they serve as a suitably sensitive assay for screening samples for the presence of neurotoxin. Spinal cords dissected from 14-15 day old foetal Sprague Dawley rats were cultured for 21 days using a modification of previously described method

(Ransom, B. R., Neale, E., Henkart, M., Bullock, P. N. and Nelson, P. G. (1977) *J. Neurophysiol.* **40**, 1132-1150 & Fitzgerald, S. C. (1989) "A dissection and tissue culture manual of the nervous system", Alan R. Liss, Inc., New York, NY). Cells were loaded with [^{3}H]-glycine for 30 minutes prior to determination of basal and potassium-stimulated release of transmitter (essentially as described in Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E. and Neale, E. A. (1996) *J. Biol. Chem.* **271**, 7694-7699). A sample of 0.2M NaOH-lysed cells was used to determine total counts, from which % release could be calculated.

In figure 3 there is shown a comparison of the inhibition curves obtained after 3 days incubation of spinal cord neurons with LH_N/A and BoNT/A. Samples of LH_N/A (●) or BoNT/A (□) were diluted in eSCN growth medium and 1ml of the appropriate concentration applied. Cells were incubated for three days prior to assessment of [^{3}H]-glycine release. The % inhibition data is calculated by relating the net stimulated [^{3}H]-glycine release to the total uptake and then expressing this as a percentage of the release obtained from control media treated cells. The data are representative of at least three experiments. The IC_{50} data determined for LH_N/A (106.2 ± 49.3nM) and BoNT/A can be used to estimate the ratio of BoNT/A to LH_N/A in the purified material. It is estimated that a maximum of 1 BoNT/A molecule per 4×10^6 LH_N/A molecules was present in the final purified material.

Example 6

<u>Removal of BoNT/A from LH_N/A preparations as assessed by mouse lethality</u>

Residual BoNT/A contamination was evaluated following intraperitoneal injection of 0.5ml of test sample in gelatine-phosphate buffer (1% (w/v) Na_2HPO_4, 0.2% (w/v) gelatine, pH 6.5-6.6) into mice. After 4 days the number of surviving animals was counted. Literature precedents (Shone

et al. 1987 & de Pavia, A. and Dolly, J. O. 1990) cite the LD_{50} to be the concentration of test sample that killed half the animals in the test group within 4 days. By this analysis, batches of purified LH_N/A were demonstrated to exhibit an LD_{50} of approximately 50μg/mouse i.e. approximately 20LD_{50}/mg. This is significantly lower than previously reported LD_{50} data for LH_N/A (6000-12000LD_{50}/mg by Shone, C. C., Hambleton, P. and Melling, J. (1987) *Eur. J. Biochem.* **167**, 175-180) and LC/A (<100LD_{50}/mg in Shone *et al.* 1987 & 10000LD_{50}/mg in de Pavia, A. and Dolly, J. O. (1990) *FEBS Lett.*, **277**, 171-174).

As an alternative method of data analysis, the mean number of survivors from different batches of LH_N/A was determined and is presented in Figure 4 (Abbreviations: IMAC; immobilised metal affinity column, ND; Not determined). These data clearly demonstrate the significantly low toxicity of the LH_N/A prepared by both exemplified methods of purification.

CLAIMS

1. A method of reducing toxicity of a toxin derivative preparation, comprising contacting said preparation with a ligand which selectively binds to the toxin but not to the toxin derivative.

2. A method according to Claim 1 wherein the ligand binds to an H_C portion of the toxin.

3. A method according to Claim 2 wherein the ligand is or comprises a metal ion which binds to the H_C portion of the toxin.

4. A method according to Claim 1 or 2 wherein the ligand is an antibody that binds to the toxin.

5. A method according to Claim 4 comprising contacting the preparation with a plurality of antibodies which selectively bind the toxin but not the toxin derivative.

6. A method of removing toxin from a toxin derivative preparation comprising contacting the preparation with a ligand according to any of Claims 1 to 5 and further comprising separating the ligand from the toxin derivative preparation.

7. A method according to Claim 6 wherein the ligand is part of or is bound to or is otherwise attached to an affinity column.

8. A method according to Claim 7 comprising adding the toxin derivative preparation to the affinity column and eluting therefrom a preparation from which toxin has been removed.

9. A method according to any of Claims 1 to 8 wherein the toxin derivative is selected from a non-toxic fragment or variant of a toxin, a non-toxic conjugate comprising a fragment or a variant of a toxin and another derivative of a toxin which is obtained directly or indirectly from native toxin.

10. A method according to Claim 9 wherein the derivative is an LH_N fragment.

11. A method according to Claim 9 wherein the derivative is a conjugate of an LH_N fragment with a targeting ligand.

12. A method according to any of Claims 1 to 11, comprising obtaining the toxin derivative by cleavage of native toxin to yield a mixture of uncleaved toxin and toxin derivative, and subjecting that mixture to a purification step to remove uncleaved toxin.

13. A method according to Claim 12 comprising purifying the mixture so as to remove uncleaved toxin by anion - exchange chromatography, cation-exchange chromatography, hydrophobic interaction chromatography or size-exclusion chromatography.

14. An affinity chromatography column, for removal of toxin from a toxin derivative preparation, wherein the column comprises a ligand that selectively binds to toxin but not to the toxin derivative.

15. A column according to Claim 14 wherein the ligand is selected from an antibody, and a toxin receptor.

16. A toxin derivative preparation comprising 1-100 ppm toxin per toxin derivative.

17. A composition comprising a derivative of a toxin and a pharmaceutically acceptable carrier, and further comprising a ligand that binds selectively to the toxin.

18. A composition according to Claim 17, comprising a conjugate of a toxin with a ligand that binds selectively to the toxin, wherein the toxin is bound non-covalently to the ligand.

19. A composition according to Claim 18 wherein the ligand is an antibody that selectively binds to the toxin.

20. A pharmaceutical composition comprising a toxin derivative or a composition according to any of Claims 16 - 19 in combination with a pharmaceutically acceptable carrier.

21. Use of an affinity chromatography column according to Claim 14 or 15 for removal of toxin from a toxin derivative preparation.

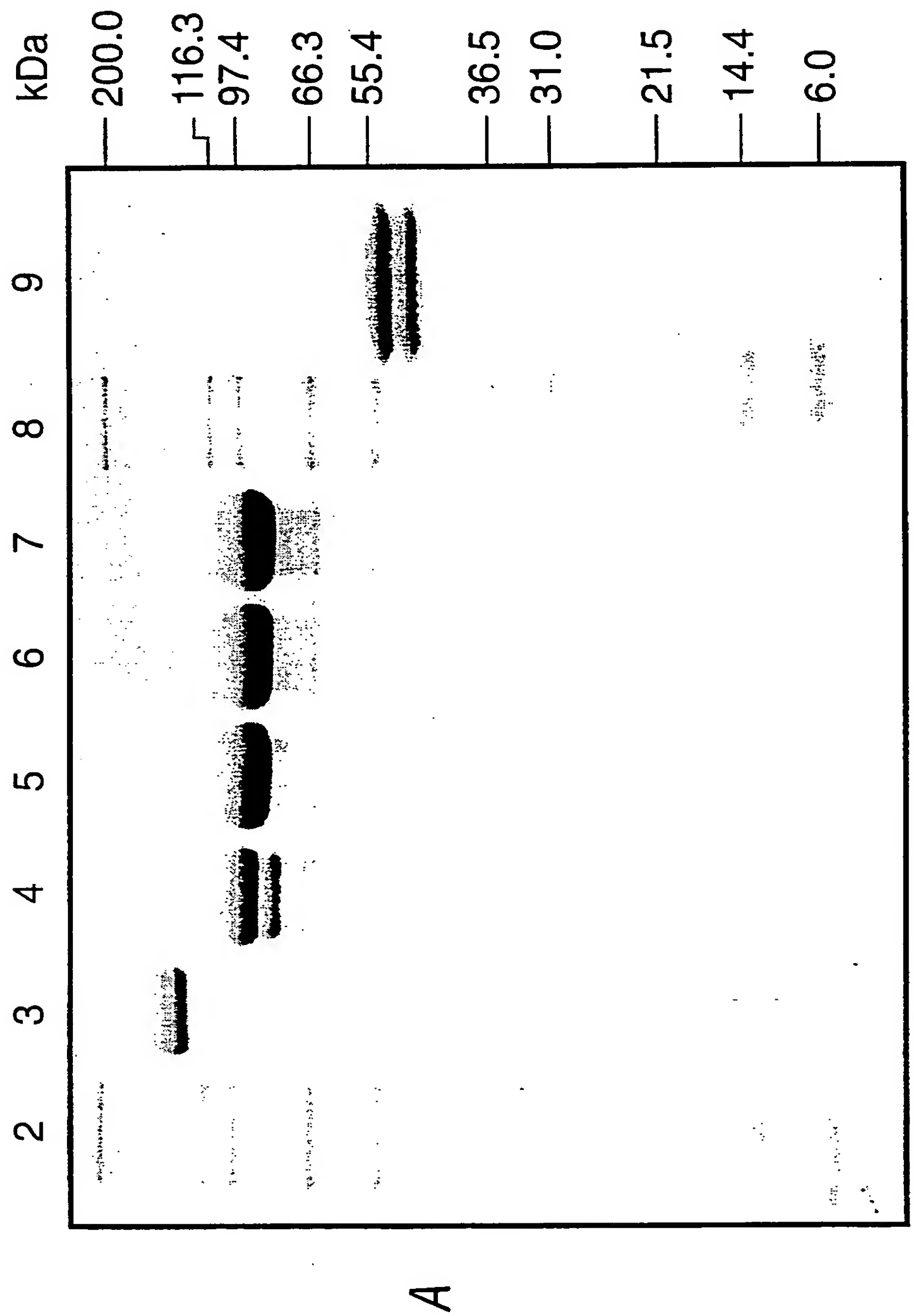
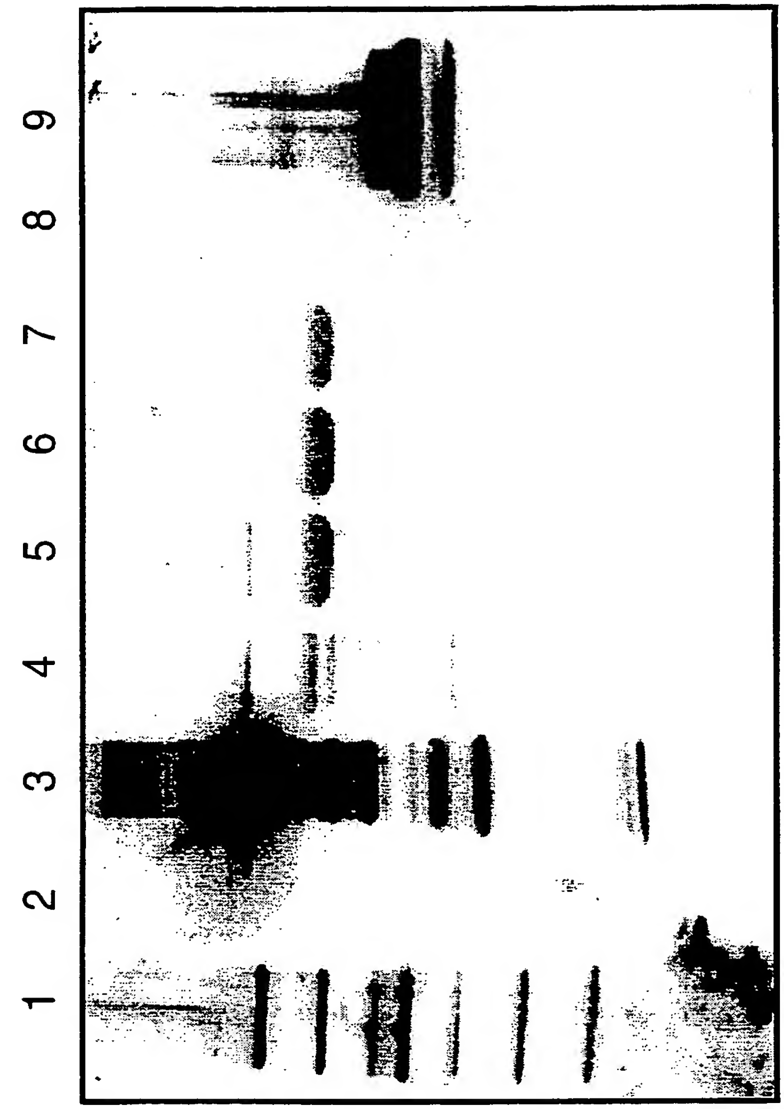


FIG. 1

SDS-PAGE analysis of LH_N/A purified by immunoaffinity approach

FIG. 1(contd.)



B

FIG. 2

Cleavage of SNAP-25 by LH_N/A

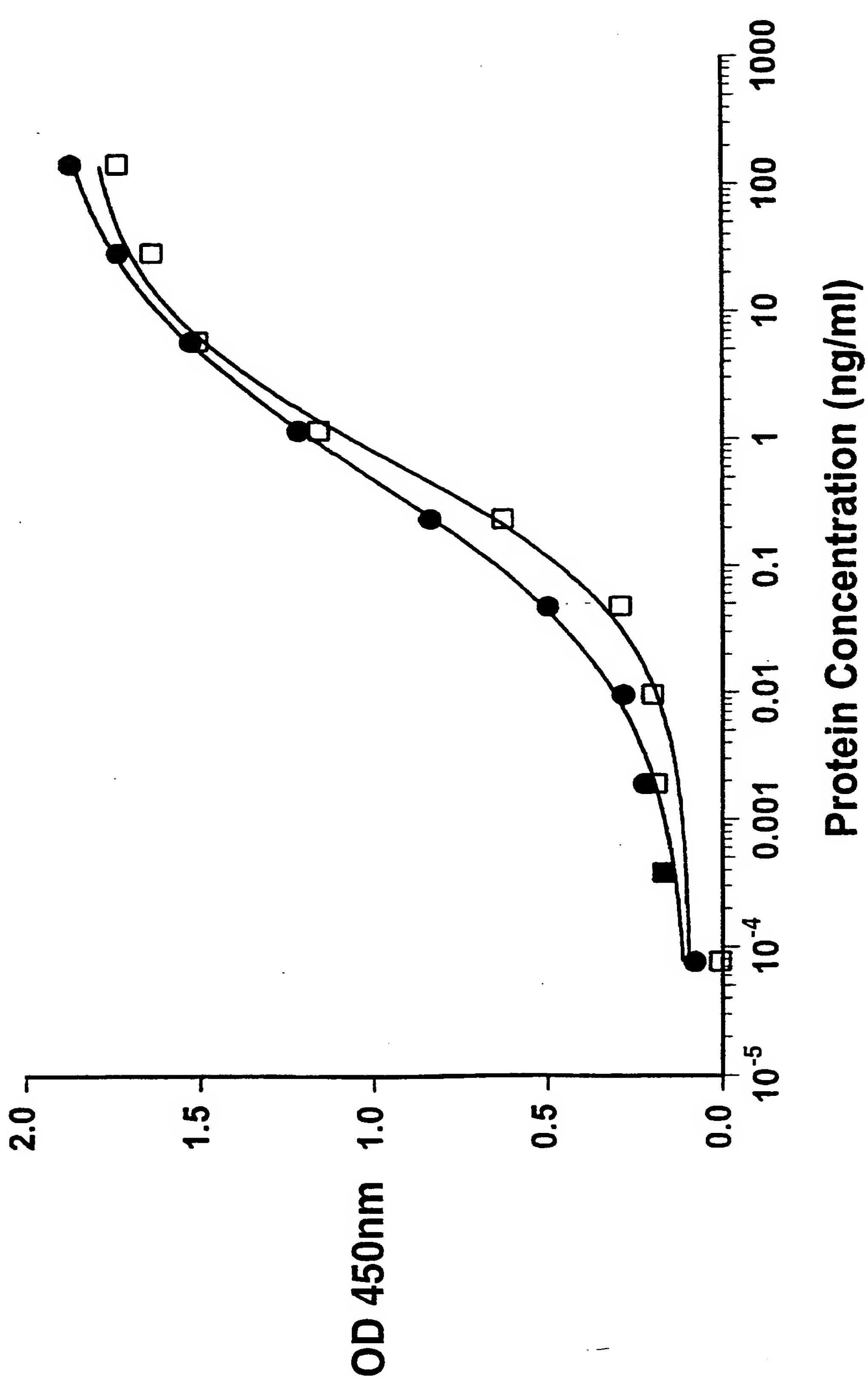


FIG. 3

Inhibition of neurotransmitter release from eSCN by LH_N/A and BoNT/A

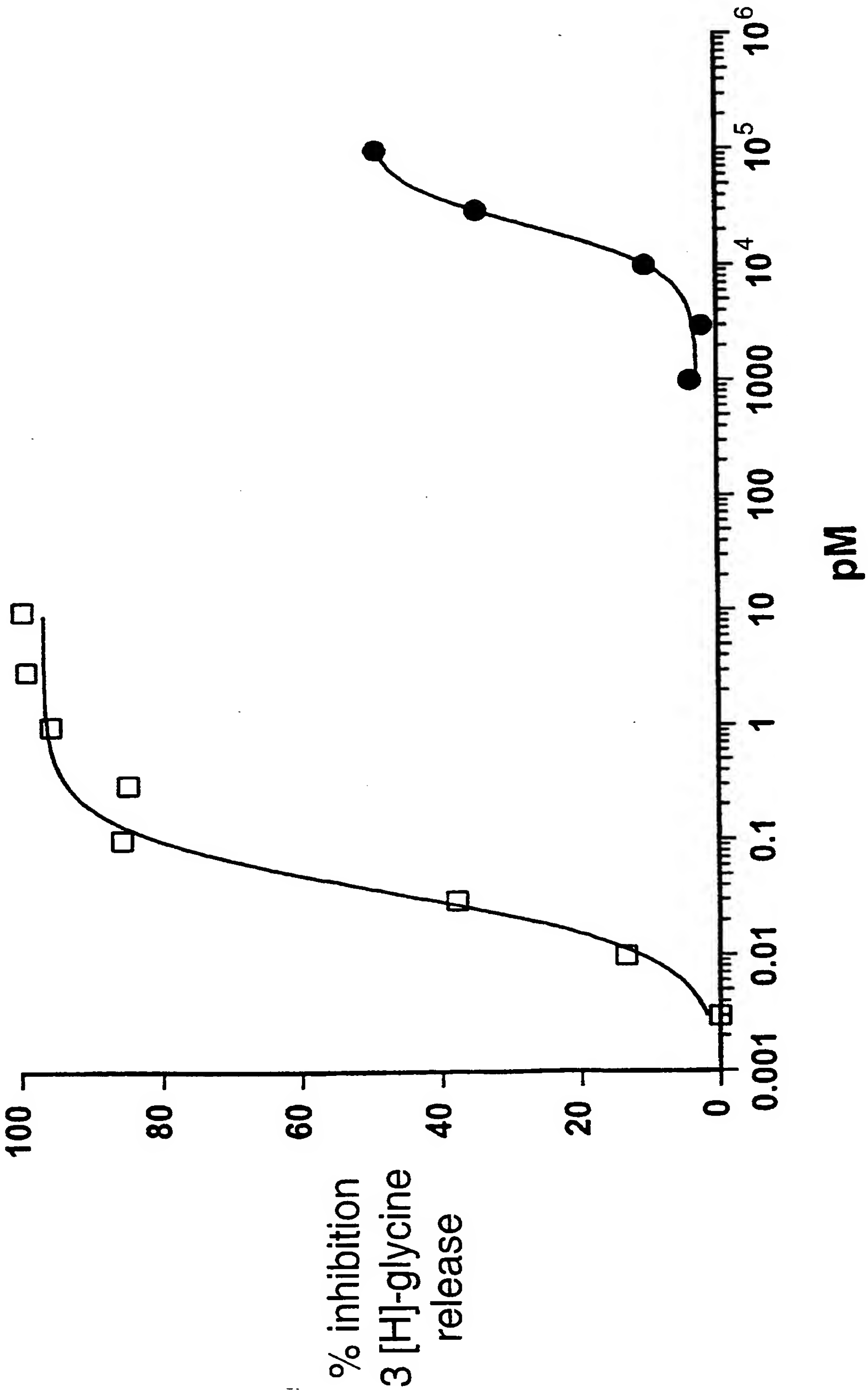


FIG. 4

Mouse toxicity data for purified vs. standard LH_N/A

	Mean number of survivors (/4)		
	50µg/mouse	20µg/mouse	5µg/mouse
Immunocolumn strategy (n=15)	1.60±0.41	3.67±0.16	ND
IMAC strategy (n=3)	1.33±0.88	3.33±0.33	ND
Mono Q® only (n=3)	0	0	1.33±1.33

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/03519

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/33 A61K39/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 601 826 A (HALPERN JANE L) 11 February 1997 (1997-02-11) column 2, line 17 - line 30 column 3, line 3 -column 7 examples 2,5 ---	1,2,4, 6-10, 12-21
Y	GIMENEZ ET AL.: "Botulinum type A neurotoxin digested with pepsin yields 132, 97, 72, 45, 42, and 18 kD fragments" JOURNAL OF PROTEIN CHEMISTRY, vol. 12, no. 3, 1993, pages 351-363, XP000986491 cited in the application abstract figure 1 page 359, left-hand column, paragraph 4 -page 359, right-hand column, paragraph 1 --- -/--	1,2,4, 6-10, 12-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2001	22/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ceder, O

1

International Application No
PCT/GB 00/03519

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE 40 37 809 A (YOUSIF YEHIA MOHAMMED) 4 June 1992 (1992-06-04) the whole document ---	1,6-8, 12-15,21
A	ZA 9 803 690 A (SYNSORB BIOTECH INC) 4 February 1999 (1999-02-04) page 1, line 3 - line 14 page 22, line 12 -page 23, line 24 ---	1,6-8,14
A	SCHIAVO G ET AL: "BOTULINUM NEUROTOXONS ARE ZINC PROTEINS" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 267, no. 33, 25 November 1992 (1992-11-25), pages 23479-23483, XP002009347 ISSN: 0021-9258 -----	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/03519

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5601826 A	11-02-1997	AU 4545393 A	24-01-1994
		WO 9400487 A	06-01-1994
DE 4037809 A	04-06-1992	NONE	
ZA 9803690 A	04-02-1999	US 6013634 A	11-01-2000